

PLANT FATTY ACID SYNTHASES AND USE IN IMPROVED METHODS FOR
PRODUCTION OF MEDIUM-CHAIN FATTY ACIDS

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INTRODUCTION

Field of Invention

The present invention is directed to genes encoding plant
10 fatty acid synthase enzymes relevant to fatty acid synthesis in
plants, and to methods of using such genes in combination with
genes encoding plant medium-chain preferring thioesterase
proteins. Such uses provide a method to increase the levels of
medium-chain fatty acids that may be produced in seed oils of
15 transgenic plants.

Background

Higher plants synthesize fatty acids via a common
metabolic pathway. In developing seeds, where fatty acids
20 attached to triglycerides are stored as a source of energy for
further germination, the fatty acid synthesis pathway is
located in the plastids. The first step is the formation of
acetyl-ACP (acyl carrier protein) from acetyl-CoA and ACP
catalyzed by a short chain preferring condensing enzyme, β -
25 ketoacyl-ACP synthase (KAS) III. Elongation of acetyl-ACP to
16- and 18- carbon fatty acids involves the cyclical action of
the following sequence of reactions: condensation with a two-

carbon unit from malonyl-ACP to form a longer β -ketoacyl-ACP (β -ketoacyl-ACP synthase), reduction of the keto-function to an alcohol (β -ketoacyl-ACP reductase), dehydration to form an enoyl-ACP (β -hydroxyacyl-ACP dehydrase), and finally reduction 5 of the enoyl-ACP to form the elongated saturated acyl-ACP (enoyl-ACP reductase). β -ketoacyl-ACP synthase I (KAS I), is primarily responsible for elongation up to palmitoyl-ACP 10 (C16:0), whereas β -ketoacyl-ACP synthase II (KAS II) is predominantly responsible for the final elongation to stearoyl-ACP (C18:0).

Genes encoding peptide components of β -ketoacyl-ACP synthases I and II have been cloned from a number of higher plant species, including castor (*Ricinus communis*) and *Brassica* species (USPN 5,510,255). KAS I activity was associated with a 15 single synthase protein factor having an approximate molecular weight of 50 kD (synthase factor B) and KAS II activity was associated with a combination of two synthase protein factors, the 50 kD synthase factor B and a 46 kd protein designated synthase factor A. Cloning and sequence of a plant gene 20 encoding a KAS III protein has been reported by Tai and Jaworski (*Plant Physiol.* (1993) 103:1361-1367).

The end products of plant fatty acid synthetase activities are usually 16- and 18-carbon fatty acids. There are, however, several plant families that store large amounts of 8- to 14- 25 carbon (medium-chain) fatty acids in their oilseeds. Recent studies with *Umbellularia californica* (California bay), a plant that produces seed oil rich in lauric acid (12:0), have

demonstrated the existence of a medium-chain-specific isozyme of acyl-ACP thioesterase in the seed plastids. Subsequent purification of the 12:0-ACP thioesterase from *Umbellularia californica* led to the cloning of a thioesterase cDNA which was 5 expressed in seeds of *Arabidopsis* and *Brassica* resulting in a substantial accumulation of lauric acid in the triglyceride pools of these transgenic seeds (USPN 5,512,482). These results and subsequent studies with medium-chain thioesterases from other plant species have confirmed the chain-length-10 determining role of acyl-ACP thioesterases during de novo fatty acid biosynthesis (T. Voelker (1996) *Genetic Engineering*, Ed. J. K. Setlow, Vol. 18, pgs. 111-133).

DESCRIPTION OF THE FIGURES

15 Figure 1. DNA and translated amino acid sequence of *Cuphea hookeriana* KAS factor B clone chKAS B-2 are provided.

Figure 2. DNA and translated amino acid sequence of *Cuphea hookeriana* KAS factor B clone chKAS B-31-7 are provided.

Figure 3. DNA and translated amino acid sequence of *Cuphea hookeriana* KAS factor A clone chKAS A-2-7 are provided.

20 Figure 4. DNA and translated amino acid sequence of *Cuphea hookeriana* KAS factor A clone chKAS A-1-6 are provided.

Figure 5. DNA and translated amino acid sequence of *Cuphea pullcherrima* KAS factor B clone cpuKAS B/7-8 are provided.

25 Figure 6. DNA and translated amino acid sequence of *Cuphea pullcherrima* KAS factor B clone cpuKAS B/8-7A are provided.

Figure 7. DNA and translated amino acid sequence of *Cuphea pullcherrima* KAS factor A clone cpuKAS A/p7-6A are provided.

Figure 8. Preliminary DNA sequence of *Cuphea pullcherrima* KAS factor A clone cpuKAS A/p8-9A is provided.

5 Figure 9. DNA and translated amino acid sequence of *Cuphea hookeriana* KASIII clone chKASIII-27 are provided.

Figure 10. The activity profile for purified cpuKAS B/8-7A using various acyl-ACP substrates is provided.

10 Figure 11. The activity profile for purified chKAS A-2-7 and chKAS A-1-6 using various acyl-ACP substrates is provided.

Figure 12. The activity profile for purified castor KAS factor A using various acyl-ACP substrates is provided.

Figure 13. The activity profile for purified castor KAS factor B using various acyl-ACP substrates is provided.

15 Figure 14. A graph showing the number of plants arranged according to C8:0 content for transgenic plants containing CpFatB1 versus transgenic plants containing CpFatB1 + chKAS A-2-7 is provided.

Figure 15. Graphs showing the %C10/%C8 ratios in transgenic 20 plants containing ChFatB2 (4804-22-357) and in plants resulting from crosses between 4804-22-357 and 5401-9 (chKAS A-2-7 plants) are provided.

Figure 16. Graphs showing the %C10 + %C8 contents in transgenic plants containing ChFatB2 (4804-22-357) and in 25 plants resulting from crosses between 4804-22-357 and 5401-9 (chKAS A-2-7 plants) are provided.

Figure 17. Graphs showing the %C10/%C8 ratios in transgenic plants containing ChFatB2 (4804-22-357) and in plants resulting from crosses between 4804-22-357 and 5413-17 (chKAS A-2-7 + CpFatB1 plants) are provided.

5 Figure 18. Graphs showing the %C10 + %C8 contents in transgenic plants containing ChFatB2 (4804-22-357) and in plants resulting from crosses between 4804-22-357 and 5413-17 (chKAS A-2-7 + CpFatB1 plants) are provided.

10 Figure 19. Graphs showing the %C12:0 in transgenic plants containing Uc FatB1 (LA86DH186) and in plants resulting from crosses with wild type (X WT) and with lines expressing Ch KAS A-2-7.

15 Figure 20. Graph showing the relative proportions of C12:0 and C14:0 fatty acids in the seeds of transgenic plants containing Uc FatB1 (LA86DH186) and in plants resulting from crosses with wild type (X WT) and with lines expressing Ch KAS A-2-7.

20 Figure 21. Graphs showing the %C18:0 in transgenic plants containing Garm FatB1 (5266) and in seeds of plants resulting from crosses with wild type (X WT) and with lines expressing Ch KAS A-2-7.

Figure 22. The activity profile of Ch KAS A in protein extracts from transgenic plants containing Ch KAS A-2-7.

Extracts were pretreated with the indicated concentrations of cerulenin.

SUMMARY OF THE INVENTION

By this invention, compositions and methods of use related to β -ketoacyl-ACP synthase (KAS) are provided. Also of interest are methods and compositions of amino acid and nucleic acid sequences related to biologically active plant synthase(s).

In particular, genes encoding KAS protein factors A and B from *Cuphea* species are provided. The KAS genes are of interest for use in a variety of applications, and may be used to provide synthase I and/or synthase II activities in transformed host cells, including bacterial cells, such as *E. coli*, and plant cells. Synthase activities are distinguished by the preferential activity towards longer and shorter acyl-ACPs as well as by the sensitivity towards the KAS specific inhibitor, cerulenin. Synthase protein preparations having preferential activity towards medium chain length acyl-ACPs are synthase I-type or KAS I. The KAS I class is sensitive to inhibition by cerulenin at concentrations as low as 1 μ M. Synthases having preferential activity towards longer chain length acyl-ACPs are synthase II-type or KAS II. The KAS enzymes of the II-type are also sensitive to cerulenin, but at higher concentrations (50 μ M). Synthase III-type enzymes have preferential activity towards short chain length acyl-ACPs and are insensitive to cerulenin inhibition.

Nucleic acid sequences encoding a synthase protein may be employed in nucleic acid constructs to modulate the amount of synthase activity present in the host cell, especially the

relative amounts of synthase I-type, synthase II-type and synthase III-type activity when the host cell is a plant host cell. In addition, nucleic acid constructs may be designed to decrease expression of endogenous synthase in a plant cell as well. One example is the use of an anti-sense synthase sequence under the control of a promoter capable of expression in at least those plant cells which normally produce the enzyme.

Of particular interest in the present invention is the coordinate expression of a synthase protein with the expression of thioesterase proteins. For example, coordinated expression of synthase factor A and a medium-chain thioesterase provides a method for increasing the level of medium-chain fatty acids which may be harvested from transgenic plant seeds.

Furthermore, coordinated expression of a synthase factor A gene with plant medium-chain thioesterase proteins also provides a method by which the ratios of various medium-chain fatty acids produced in a transgenic plant may be modified. For example, by expression of a synthase factor A, it is possible to increase the ratio of C10/C8 fatty acids which are produced in plant seed oils as the result of expression of a thioesterase having activity on C8 and C10 fatty acids.

DETAILED DESCRIPTION OF THE INVENTION

A plant synthase factor protein of this invention includes a sequence of amino acids or polypeptide which is required for catalyzation of a condensation reaction between an acyl-ACP

having a chain length of C₂ to C₁₆ and malonyl-ACP in a plant host cell. A particular plant synthase factor protein may be capable of catalyzing a synthase reaction in a plant host cell (for example as a monomer or homodimer) or may be one component 5 of a multiple peptide enzyme which is capable of catalyzing a synthase reaction in a plant host cell, i.e. one peptide of a heterodimer.

Synthase I (KAS I) demonstrates preferential activity towards acyl-ACPs having shorter carbon chains, C₂-C₁₄ and is 10 sensitive to inhibition by cerulenin at concentrations of 1 μ M. Synthase II (KAS II) demonstrates preferential activity towards acyl-ACPs having longer carbon chains, C₁₄-C₁₆, and is inhibited by concentrations of cerulenin (50 μ M). Synthase III demonstrates preferential activity towards acyl-CoAs having 15 very short carbon chains, C₂ to C₆, and is insensitive to inhibition by cerulenin.

Synthase factors A and B, and synthase III proteins obtained from medium-chain fatty acid producing plant species of the genus *Cuphea* are described herein. As described in the 20 following Examples, synthase A from *C. hookeriana* is naturally expressed at a high level and only in the seeds. *C. hookeriana* synthase B is expressed at low levels in all tissues examined. Expression of synthase A and synthase B factors in *E. coli* and 25 purification of the resulting proteins is employed to determine activity of the various synthase factors. Results of these analyses indicate that synthase factor A from *Cuphea hookeriana* has the greatest activity on 6:0-ACP substrates, whereas

synthase factor B from *Cuphea pullcherrima* has greatest activity on 14:0-ACP. Similar studies with synthase factors A and B from castor demonstrate similar activity profiles between the factor B synthase proteins from *Cuphea* and castor. The 5 synthase A clone from castor, however, demonstrates a preference for 14:0-ACP substrate.

Expression of a *Cuphea hookeriana* KAS A protein in transgenic plant seeds which normally do not produce medium-chain fatty acids does not result in any detectable 10 modification of the fatty acid types and contents produced in such seeds. However, when *Cuphea hookeriana* KAS A protein is expressed in conjunction with expression of a medium-chain acyl-ACP thioesterase capable of providing for production of C8 and C10 fatty acids in plant seed oils, increases in the levels 15 of medium-chain fatty acids over the levels obtainable by expression of the medium-chain thioesterase alone are observed. In addition, where significant amounts of C8 and C10 fatty acids are produced as the result of medium-chain thioesterase expression, co-expression of a *Cuphea* KAS A protein also 20 results in an alteration of the proportion of the C8 and C10 fatty acids that are obtained. For example, an increased proportion of C10 fatty acids may be obtained by co-expression of *Cuphea hookeriana* ChFatB2 thioesterase and a chKAS A synthase factor proteins.

25 Furthermore, when *Cuphea hookeriana* KAS A protein is expressed in conjunction with expression of a medium-chain acyl-ACP thioesterase capable of providing for production of

C12 fatty acids in plant seed oils, increases in the levels of medium-chain fatty acids over the levels obtainable by expression of the medium-chain thioesterase alone are also observed. In addition, where significant amounts of C12 and C14 5 fatty acids are produced as the result of medium-chain thioesterase expression, co-expression of a *Cuphea* KAS A protein also results in an alteration of the proportion of the C12 and C14 fatty acids that are obtained. For example, an increased proportion of C12 fatty acids may be obtained by co- 10 expression of *Uc* FatB1 thioesterase and a chKAS A synthase factor proteins.

However, when *Cuphea hookeriana* KAS A protein is expressed in conjunction with the expression of a long-chain acyl-ACP thioesterase capable of providing for production of C18 and 15 C18:1 fatty acids in plant seed oils, no effect on the production of long chain fatty acids was observed. Furthermore, when plants transformed to express a long chain acyl-ACP thioesterase from mangosteen (*GarmFatA1*, U.S. Patent Application No. 08/440,845), which preferentially hydrolyzes 20 C18:0 and C18:1 fatty acyl-ACPs, are crossed with nontransformed control plants, a significant reduction in the levels of C18:0 is obtained. Similar reductions are also observed in the levels of C18:0 in the seeds of plants resulting from crosses between plants transformed to express 25 the *GarmFatA1* and plants expressing the *Cuphea hookeriana* KAS A protein.

Thus, the instant invention provides methods of increasing and/or altering the medium-chain fatty acid compositions in transgenic plant seed oils by co-expression of medium-chain acyl-ACP thioesterases with synthase factor proteins.

5 Furthermore, various combinations of synthase factors and medium-chain thioesterases may be achieved depending upon the particular fatty acids desired. For example, for increased production of C14 fatty acids, synthase protein factors may be expressed in combination with a C14 thioesterase, for example 10 from *Cuphea palustris* or nutmeg may be employed (WO 96/23892). In addition, thioesterase expression may be combined with a number of different synthase factor proteins for additional effects on medium-chain fatty acid composition.

Synthases of use in the present invention include modified 15 amino acid sequences, such as sequences which have been mutated, truncated, increased and the like, as well as such sequences which are partially or wholly artificially synthesized. The synthase protein encoding sequences provided herein may be employed in probes for further screening or used 20 in genetic engineering constructs for transcription or transcription and translation in host cells, especially plant host cells. One skilled in the art will readily recognize that antibody preparations, nucleic acid probes (DNA and RNA) and the like may be prepared and used to screen and recover 25 synthases and/or synthase nucleic acid sequences from other sources. Typically, a homologously related nucleic acid sequence will show at least about 60% homology, and more

preferably at least about 70% homology, between the *R. communis* synthase and the given plant synthase of interest, excluding any deletions which may be present. Homology is determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions.

Recombinant constructs containing a nucleic acid sequence encoding a synthase protein factor or nucleic acid sequences encoding a synthase protein factor and a medium-chain acyl-ACP thioesterase may be prepared by methods well known in the art.

Constructs may be designed to produce synthase in either prokaryotic or eukaryotic cells. The increased expression of a synthase in a plant cell, particularly in conjunction with expression of medium-chain thioesterases, or decreasing the amount of endogenous synthase observed in plant cells are of special interest.

Synthase protein factors may be used, alone or in combination, to catalyze the elongating condensation reactions of fatty acid synthesis depending upon the desired result. For example, rate influencing synthase activity may reside in synthase I-type, synthase II-type, synthase III-type or in a combination of these enzymes. Furthermore, synthase activities may rely on a combination of the various synthase factors described herein.

Constructs which contain elements to provide the transcription and translation of a nucleic acid sequence of interest in a host cell are "expression cassettes". Depending upon the host, the regulatory regions will vary, including

regions from structural genes from viruses, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be 5 employed. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *E. coli*, *B. subtilis*, *Saccharomyces cerevisiae*, including genes such as β -galactosidase, T7 polymerase, trp-lac (lac), trp E and the like.

10 An expression cassette for expression of synthase in a plant cell will include, in the 5' to 3' direction of transcription, a transcription and translation initiation control regulatory region (also known as a "promoter") functional in a plant cell, a nucleic acid sequence encoding a 15 synthase, and a transcription termination region. Numerous transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the desaturase structural gene. Among transcriptional initiation regions used for plants are 20 such regions associated with cauliflower mosaic viruses (35S, 19S), and structural genes such as for nopaline synthase or mannopine synthase or napin and ACP promoters, etc. The transcription/ translation initiation regions corresponding to such structural genes are found immediately 5' upstream to the 25 respective start codons. Thus, depending upon the intended use, different promoters may be desired.

Of special interest in this invention are the use of promoters which are capable of preferentially expressing the synthase in seed tissue, in particular, at early stages of seed oil formation. Examples of such seed-specific promoters include

5 the region immediately 5' upstream of a napin or seed ACP genes such as described in USPN 5,420,034, desaturase genes such as described in Thompson *et al* (*Proc. Nat. Acad. Sci.* (1991) 88:2578-2582), or a Bce-4 gene such as described in USPN 5,530,194. Alternatively, the use of the 5' regulatory region

10 associated with the plant synthase structural gene, i.e., the region immediately 5' upstream to a plant synthase structural gene and/or the transcription termination regions found immediately 3' downstream to the plant synthase structural gene, may often be desired. In general, promoters will be

15 selected based upon their expression profile which may change given the particular application.

In addition, one may choose to provide for the transcription or transcription and translation of one or more other sequences of interest in concert with the expression or

20 anti-sense of the synthase sequence, particularly medium-chain plant thioesterases such as described in USPN 5,512,482, to affect alterations in the amounts and/or composition of plant oils.

When one wishes to provide a plant transformed for the

25 combined effect of more than one nucleic acid sequence of interest, a separate nucleic acid construct may be provided for each or the constructs may both be present on the same plant

transformation construct. The constructs may be introduced into the host cells by the same or different methods, including the introduction of such a trait by crossing transgenic plants via traditional plant breeding methods, so long as the 5 resulting product is a plant having both characteristics integrated into its genome.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformed 10 cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species into which the expression construct or 15 components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

The manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method 20 which provides for efficient transformation may be employed. Various methods for plant cell transformation include the use of Ti- or Ri-plasmids, microinjection, electroporation, liposome fusion, DNA bombardment or the like. In many instances, it will be desirable to have the construct bordered 25 on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or

A. *rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation.

The expression constructs may be employed with a wide variety of plant life, particularly plant life involved in the 5 production of vegetable oils. These plants include, but are not limited to rapeseed, peanut, sunflower, safflower, cotton, soybean, corn and oilseed palm.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed 10 *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to 15 rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

The invention now being generally described, it will be more readily understood by reference to the following examples 20 which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

25 **Example 1 Cuphea KAS Factor A and B Gene Cloning**

Total RNA isolated from developing seeds of *Cuphea hookeriana* and *Cuphea pullcherrima* was used for cDNA synthesis

in commercial 1-based cloning vectors. For cloning each type of KAS gene, approximately 400,000-500,000 unamplified recombinant phage were plated and the plaques transferred to nitrocellulose. For KAS factor B cloning from *C. hookeriana*, a 5 mixed probe containing *Brassica napus* KAS factor B and *Ricinus communis* (Castor) KAS factor B radiolabeled cDNA's was used. Similarly, a mixed probe containing *Brassica napus* KAS factor A and *Ricinus communis* KAS factor A cDNA clones was used to obtain *C. hookeriana* KAS factor A genes. For KASIII, a 10 spinach KASIII cDNA clone obtained from Dr. Jan Jaworski was radiolabeled and used as a probe to isolate a KASIII clone from *C. hookeriana*. For KAS B and KAS A cloning from *C. pullcherrima*, *C. hookeriana* KAS B and KAS A genes chKAS B-2 and chKAS A-2-7 (see below) were radiolabeled and used as probes. 15 DNA sequence and translated amino acid sequence for *Cuphea* KAS clones are provided in Figures 1-9. *Cuphea hookeriana* KAS factor B clones chKAS B-2 and chKAS B-31-7 are provided in Figures 1 and 2. Neither of the clones is full length. *Cuphea hookeriana* KAS Factor A clones chKAS A-2-7 and chKAS A-1-6 are 20 provided in Figures 3 and 4. chKAS A-2-7 contains the entire encoding sequence for the KAS factor protein. Based on comparison with other plant synthase proteins, the transit peptide is believed to be represented in the amino acids encoded by nucleotides 125-466. chKAS A-1-6 is not a full 25 length clone although some transit peptide encoding sequence is present. Nucleotides 1-180 represent transit peptide encoding

sequence, and the mature protein encoding sequence is believed to begin at nucleotide 181.

Cuphea pullcherrima KAS factor B clones cpuKAS B/7-8 and cpuKAS B/8-7A are provided in Figures 5 and 6. Both of the 5 clones contain the entire encoding sequences for the KAS factor B proteins. The first 35 amino acids of cpuKAS B/7-8 are believed to represent the transit peptide, with the mature protein encoding sequence beginning at nucleotide 233. The first 39 amino acids of cpuKAS B/8-7A are believed to represent 10 the transit peptide, with the mature protein encoding sequence beginning at nucleotide 209. *Cuphea pullcherrima* KAS factor A clones cpuKAS A/p7-6A and cpuKAS A-p8-9A are provided in Figures 7 and 8. Both of the clones contain the entire encoding sequences for the KAS factor A proteins. Translated 15 amino acid sequence of cpuKAS A/p7-6A is provided. The mature protein is believed to begin at the lysine residue encoded 595-597, and the first 126 amino acids are believed to represent the transit peptide. The DNA sequence of KAS A clone cpuKAS A-p8-9A is preliminary. Further analysis will be conducted to 20 determine final DNA sequence and reveal the amino acid sequence encoded by this gene.

DNA and translated amino acid sequence of *Cuphea hookeriana* KASIII clone chKASIII-27 is provided in Figure 9. The encoding sequence from nucleotides 37-144 of chKASIII-27 25 are believed to encode a transit peptide, and the presumed mature protein encoding sequence is from nucleotides 145-1233.

Deduced amino acid sequence of the *C. hookeriana* KAS factor B and KAS factor A cDNA's reveals strong homology to the *Brassica napus* and *Ricinus communis* clones previously reported. The *C. hookeriana* KAS factor B clone is more homologous to the 5 *Ricinus* and *Brassica* KAS factor B clones (94% and 91% respectively) than it is to the *Ricinus* and *Brassica* KAS factor A clones (60% for both). Furthermore, the *C. hookeriana* KAS factor A clone is more homologous to the *Ricinus* and *Brassica* KAS factor A clones (85% and 82% respectively) than it is the 10 *Ricinus* and *Brassica* KAS factor B clone (60% for both). The *C. hookeriana* KAS factor B cDNAs designated as chKAS B-2 and chKAS B-31-7 are 96% identical within the mature portion of the polypeptide. Similarly, the deduced amino acid sequence of the mature protein regions of the *C. hookeriana* KAS factor A clones 15 chKAS A-2-7 and chKAS A-1-6 are 96% identical. The *C. pullcherrima* KAS clones also demonstrate homology to the *R. communis* and *Brassica napus* KAS clones. The mature protein portion of all of the KAS factor A family members in the different *Cuphea* species are 95% identical. Similarly the 20 mature protein portion of the KAS factor B genes in *Cuphea* are also 95-97% identical with each other. However there is only approximately 60% sequence identity between KAS factor B and KAS factor A clones either within the same or different species of *Cuphea*.

Example 2 Levels and Patterns of Expression

To examine tissue specificity of KAS expression in *Cuphea hookeriana*, Northern blot analysis was conducted using total RNA isolated from seed, root, leaf and flower tissue. Two 5 separate but identical blots were hybridized with either chKAS B-31-7 or chKAS A-2-7 coding region probes. The data from this RNA blot analysis indicate that KAS B is expressed at a similar level in all tissues examined, whereas KAS A expression is detected only in the seed. These results also demonstrate a 10 different level of expression for each of the synthases. KAS A is an abundant message, whereas KAS B is expressed at low levels. Furthermore, even under highly stringent hybridization conditions (65°C, 0.1 X SSC, 0.5% SDS), the KAS A probe hybridizes equally well with two seed transcripts of 2.3 15 and 1.9 kb. The larger hybridizing band is likely the transcript of the KAS A-2-7 gene since the size of its cDNA is 2046bp, and the number of clones obtained from cDNA screening corresponds well with the apparent mobility of the mRNA and its abundance on the blot.

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Example 3 Expression of Plant KAS Genes in E.coli

DNA fragments encoding the mature polypeptide of the *Cuphea hookeriana* KAS A cDNAs and the *Cuphea pullcherrima* KAS B cDNAs were obtained by PCR and cloned into a QIAexpress 25 expression vector (Qiagene). Experimental conditions for maximum level of expression were determined for all of these clones and the parameters for highest level of soluble fraction

were identified. Cells are grown in ECLB media containing 1M sorbitol and 2.5 mM betaine overnight and subcultured as a 1:4 dilution in the same medium. Cells are then grown for 2 hours (to approximately .6-.8 O.D.) and induced with 0.4 mM IPTG and 5 allowed to grow for 5 more hours.

Enzyme activity of the affinity purified recombinant enzymes obtained from over-expression of the chKAS A-2-7 and cpuKAS B/8-7A clones was measured using a wide range of acyl-ACP substrates (6:0- to 16:1-ACP). The activity profile for 10 cpuKAS B/8-7A is provided in Fig.10. The data demonstrate that the enzyme is active with all acyl-ACP substrates examined, although activity on 6:0 to 14:0-ACP substrates is substantially greater than the activity on 16:0 and 16:1 substrates.

15 The activity profile of the *C. hookeriana* KAS A clones chKAS A-2-7 and chKAS A-1-6 is provided in Figure 11. The *C. hookeriana* KAS A clones are most active with C:6, and have the least activity with C:16:0 substrates. However, the activity of this clone on even the preferred C6:0 substrate is 50 fold 20 lower than the activity of the *C. pullcherrima* KAS B clones.

A fragment containing the mature protein encoding portion of a *R. communis* KAS factor A clone was also cloned into a QIAexpress expression vector, expressed in *E. coli* and the enzyme affinity purified as described above. The activity 25 profile for castor KAS A is provided in Figure 12. Highest activity is observed with C14:0 substrates, although some activity is also seen with C6:0 and C16:1. In comparison, the

activity profile obtained from purified *R. communis* KAS factor B also using the QIAexpress expression system is provided in Figure 13. The KAS B clone demonstrates substantially higher levels of activity (10 fold and higher) than the *R. communis* KAS A clone. The preference of the KAS factor B for 6:0- to 14:0-ACP substrates is consistent with the previous observations that this protein provides KAS I activity.

Example 4 KAS and TE Expression in Transgenic Seed

10 Both the CpFatB1 (*C. hookeriana* thioesterase cDNA; Dehesh et al. (1996) *Plant Physiol.* 110:203-210) and the chKAS A-2-7 were PCR amplified, sequenced, and cloned into a napin expression cassette. The napin/cp FatB1 and the napin/KAS A-2-7 fusions were ligated separately into the binary vector 15 pCGN1558 (McBride and Summerfelt (*Pl. Mol. Biol.* (1990) 14:269-276) and transformed into *A. tumefaciens*, EHA101. The resulting CpFatB1 binary construct is pCGN5400 and the chKAS A-2-7 construct is pCGN5401. *Agrobacterium* mediated transformation of a *Brassica napus* canola variety was carried 20 out as described by Radke et al. (*Theor. Appl. Genet.* (1988) 75:685-694; *Plant Cell Reports* (1992) 11:499-505). Several transgenic events were produced for each of the pCGN5400 and pCGN5401 constructs.

25 A double gene construct containing a napin/cpFatB1 expression construct in combination with a napin/chKAS A-2-7 expression construct was also assembled, ligated into a binary vector and used for co-cultivation of a canola *Brassica*

variety. The binary construct containing the chFatB1 and chKAS A-2-7 expression constructs is pCGN5413.

Fatty acid analysis of 26 transgenic lines containing chKAS A-2-7 (5401 lines) showed no significant changes in the 5 oil content or profile as compared to similar analyses of wild type canola seeds of the transformed variety.

Fatty acid analysis of 36 transgenic lines containing cpFatB1 (5400 lines) showed increased levels of C:8 and C:10 in transgenic seeds. The highest level of C:8 observed in a pool 10 seed sample was 4.2 mol%. The C:10 levels were between 30 and 35% of the C:8 content. Fatty acid analysis of 25 transgenic lines containing the TE/KAS A tandem (5413 lines) demonstrated an overall increase in both C:8 and C:10 levels relative to those observed with TE containing lines (5400) alone. In lines 15 containing the cpFatB1 construct alone, the average level of C:8 average were 1.5 mol%, whereas the C:8 average levels in TE/KAS A tandem containing lines was 2.37 mol%. The ratio of C:8 to C:10 remained constant in both populations. The number of transgenic events relative to the C:8 content are presented 20 in Figure 14. These data show that the transgenic events with tandem TE/KAS A construct yield more lines with higher levels of C:8 than those events with single TE construct. For example, several lines containing nearly 7 mole% C8 were obtained with the TE/KAS A pCGN5413 construct, whereas the 25 highest C8 containing line from the pCGN5400 TE alone transformation contained 4.2 mole% C8.

Half seed analysis of the T3 generation of transgenic canola plants expressing a ChFatB2 (*C. hookeriana* thioesterase; Dehesh et al. (1996) *The Plant Journal* 9:167-172) indicate that these plant can accumulate up to 22 weight% (33 mol%) of 8:0 and 10:0 fatty acids (4804-22-357). Segregation analysis shows that these transformants contain two loci and that they are now homozygous. Selected plants grown from these half seeds were transferred into the greenhouse and later crossed with T1 transformants that had been transformed with either *Cuphea hookeriana* KAS A (5401) alone or KAS A/CpFatB1 double constructs (5413).

Fatty acid analysis of several events resulting from the crosses between transgenic lines containing ChFatB2 (4804-22-357) and chKAS A-2-7 (5401-9), reveal an increase in the ratio of C:10/C:8 levels (Figure 15). This C:10/C:8 ratio in nearly all of the transgenic events containing ChFatB2 TE alone fluctuates between 3 and 6, whereas in the F1 generation of transgenic containing both the TE and the KAS A-2-7, the ratio can be as high as 22. This increase in C:10 levels is accompanied by an increase in the total C:8 and C:10 content (Figure 16). The sum of the C:8 and C:10 fatty acids in the heterozygous F1 lines is as high as those in the homozygous parent line (4804-22-357), whereas the heterozygous lines usually contain substantially less C:8 and C:10 than the homozygous lines.

Similar results were observed in F1 generation seeds resulting from crosses performed between 4804-22-357 (ChFatB2)

and the 5413-17 event (CpFatB1 and chKAS A-2-7 tandem). Levels of C:8 and C:10 in the 5413-17 line were 6.3 and 2.8 mol% respectively. Data presented in Figure 17 show that there is shift towards C:10 fatty acids as was observed with the 4804-5 22-357 (ChFatB2) x 5401-9 (chKAS A-2-7) crosses. Furthermore, Figure 18 indicates the presence of two separate populations of heterozygotes. Those containing approximately 9-11 weight percent C:10 + C:8 are believed to represent offspring containing a single copy of the ChFatB1 TE gene and no copies 10 of the CpFatB1 and chKAS A genes from 5413. Those plants containing approximately 15-20 weight percent C:10 + C:8 are believed to represent the heterozygotes containing a single 15 ChFatB1 TE gene as well as the CpFatB1 and chKAS A genes from 5413. Thus, the level of the C:10 + C:8 fatty acids does not decrease to 50% of that detected in parent lines when a copy of the ChKAS A gene is present.

To further characterize the chain length specificity of the *Cuphea hookeriana* KAS A enzyme, crosses between transgenic *Brassica napus* lines containing a California Bay (*Umbellularia californica*) 12:0 specific thioesterase, Uc FatB1 (USPN 20 5,344,771) and chKAS A-2-7 (5401-9) were made. Half seed analysis of transgenic plants containing Uc fatB1 have previously indicated that these plants can accumulate up to 52 mol% C12:0 in the seed oil of homozygous dihaploid lines 25 (LA86DH186). Crosses between the line LA86DH186 and untransformed control *Brassica* demonstrated a decrease in the C12:0 levels.

However, crosses between LA86DH186 and the 5401-9 hemizygous line led to an accumulation of up to 57 mol% C12:0 in the seed oil of F1 progeny (Figure 19). Interestingly, in crosses with LA86DH186 x untransformed control line and 5 LA86DH186 x 5401-9, levels of C14:0 in the seeds of the F1 progeny decreased to 50% of the levels obtained in homozygous LA86DH186 lines (Figure 20). Furthermore, increases in the proportion of C12:0 fatty acid resulted in a substantial 10 decline in the proportions of all the long-chain fatty acyl groups (C16:0, C18:0, C18:2, and C18:3). These results indicate that the ChKAS A-2-7 is an enzyme with substrate 15 specificity ranging from C6:0 to C10:0-ACP, and that its over-expression ultimately reduces the longer chain acyl-ACP pools.

Further evidence is obtained in support of the chain length specificity of the ChKAS A-2-7 in crosses of the 5401-9 line with a transgenic line (5266) expressing an 18:1/18:0 TE from *Garcinia mangostana* (GarmFatA1, US patent application No. 08/440,845). Transgenic *Brassica* line 5266 has been shown to accumulate up to 24 mol% C18:0 in the seed oil of homozygous 20 lines (Figure 21). However, in the seed oil of F1 progeny of crosses between 5266 and 5401-9 levels of C18:0 were reduced to approximately 12 mol%. Furthermore, levels of C16:0 generated from these crosses was similar to the levels obtained from the seed oil of nontransgenic control plants.

Example 5 *In vitro* Analysis of Plant KAS Enzymes

Seed extracts were prepared from developing seeds of nontransgenic controls or transgenic *Brassica* expressing chKAS A-2-7 as described in Slabaugh *et al.* (*Plant Journal*, 1998 in press) and Leonard *et al.* (*Plant Journal*, 1998, in press). *In vitro* fatty acid synthesis assays were performed as described by Post-Beittenmiller (*J. Biol. Chem.* (1991), 266:1858-1865). Extracts were concentrated by ammonium sulfate precipitation and desalting using P-6 columns (Bio-Rad, Hercules, CA).

Reactions (65 μ l) contained 0.1M Tris/HCl (pH 8.0), 1 mM dithiothreitol, 25 mM recombinant spinach ACPI, 1 mM NADH, 2 mM NADPH, 50 μ M malonyl-CoA, 10 μ M [1- 14 C]acetyl-CoA (50 mCi/mmol), 1mg/ml BSA, and 0.25 mg/ml seed protein. Selected seed extracts were preincubated with cerulenin at 23°C for 10 min.

Reaction products were separated on an 18% acrlamide gel containing 2.25M urea, electroblotted onto to nitrocellulose and quntitated by phosporimaging using Image QuaNT software (Molecular Dynamics, Sunnyvale, CA). Authentic acyl-ACPs were run in parallel, immunoblotted and finally detected by anti-ACP serum to confirm fatty acid chain lengths.

The results (Figure 22) indicate that the fatty acid synthesis capabilities of transgenic *Brasica* (5401-9) seed extracts was greater than that obtained from in the nontransgenic controls as measured by the relative abundance of C8:0- and C10:0-ACP at all time points tested. In addition, pretreatment of the extracts with cerulenin, markedly reduced the synthesis of longer chain fatty acids in both the

transgenic and nontransgenic control seed extracts. However, the extension of the spinach-ACP was much less inhibited in the seed extracts from the transgenic lines than in the seed extracts of nontransgenic control *Brassica*.

5 These data further support that Ch KAS A-2-7 is a condensing enzyme active on medium chain acyl-ACPs, and that expression of this enzyme in plants results in enlarged substrate pools to be hydrolyzed by medium-chain specific thioesterases. Furthermore, these data suggest that chKAS A-2-
10 7 also is a cerulenin-resistant condensing enzyme.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All
15 publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in
20 some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.